

Mitogenomic analysis reveals two cryptic lineages and provides insights into the historical biogeography of the genus *Pseudohynobius* (Caudata, Hynobiidae)

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<https://zoobank.org/8E93D126-B5F5-4AF3-9BF3-E85F322B4B91>

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Academic editor: Umilaela Arifin ♦ Received 21 March 2025 ♦ Accepted 17 July 2025 ♦ Published 2 September 2025

Abstract

The genus *Pseudohynobius* exhibits a west-to-east distribution across southwestern China, spanning diverse mountain ranges and elevations. However, knowledge regarding the diversity, phylogeny, and evolutionary history of this genus remains limited. In this study, based on the concept of phylogenetic species, we identified eight phylogenetic species, including two cryptic lineages. Divergence time estimation revealed that *Pseudohynobius* originated approximately 14.57 million years ago (Ma), with interspecific divergence mainly occurring between 8.0 and 1.4 Ma. Biogeographic analysis indicated that its most recent common ancestor likely inhabited the Hengduan–Dalou Mountains region in southwestern China around 8.62 Ma and that the Dalou Mountains served as a source of outward dispersal and a key region for speciation between 11 and 4 Ma. The lineage divergence dynamics of this genus are coupled with orogenic movements and paleoclimatic shifts, which may have been the primary drivers of its historical diversification. This study underscores the urgency of conducting further surveys and taxonomic studies to avoid underestimating the diversity of this critically endangered genus.

Key Words

biogeography, conservation, cryptic species, molecular dating, phylogeny, *Pseudohynobius*

Introduction

The diverse natural landscapes of southern China have fostered exceptionally high species diversity, establishing it as a biodiversity hotspot, with many species being endemic to the region. Despite extensive efforts by taxonomists, the recent discovery of numerous new and cryptic species suggests that biodiversity may still be underestimated (Jiang et al. 2024; Xu et al. 2024). Previous large-scale studies have linked patterns of diversity to orogenic movements and climatic shifts since the Miocene, particularly in fish (Wen et al. 2022; Luo et al. 2024; Luo et

al. 2025), amphibians (Che et al. 2010; Luo et al. 2022; Luo et al. 2023), reptiles (Guo et al. 2020), and mammals (Feijó et al. 2022; Wang et al. 2022). However, these studies focus on broad temporal and spatial scales, leaving speciation in small areas poorly understood, especially regarding the role of geographic barriers in speciation.

The Chinese endemic genus *Pseudohynobius* Fei & Yang, 1983 (Caudata, Hynobiidae) is narrowly and intermittently distributed in the mountainous regions of southern China, including Sichuan, Guizhou, Chongqing, Hubei, and Hunan (Fei et al. 2006; Fei and Ye 2016; Amphibia-China 2025), providing an ideal model for studying the

effects of geographic isolation on speciation. Although the taxonomic placement of the genus remains controversial—specifically whether *Pseudohynobius puxiongensis* (Fei & Ye, 2000) should be treated as the monotypic genus *Protohynobius* Fei & Ye, 2000 (Fei and Ye 2000; Fei et al. 2006; Fei and Ye 2016; Jia et al. 2021) or placed within *Pseudohynobius* as a basal lineage (Peng et al. 2010; Zheng et al. 2011; Yang et al. 2013; Chen et al. 2015)—we follow the latter treatment in accordance with previous studies (AmphibiaChina 2025; Frost 2025). Currently, only six species within this genus have been recorded, inhabiting high-altitude cold pools and surrounding humus-rich environments (Fei and Ye 2016), namely *Pseudohynobius flavomaculatus* (Hu & Fei, 1978), *Pseudohynobius guizhouensis* Li, Tian & Gu, 2010, *Pseudohynobius jinbo* Wei, Xiong & Zeng, 2009, *Pseudohynobius kuankuoshuiensis* Xu & Zeng, 2007, *P. puxiongensis*, and *Pseudohynobius shuichengensis* Tian, Gu, Li, Sun & Li, 1998. Additionally, the conservation status of these species is of considerable concern, as all have been listed in the IUCN Red List of Threatened Species 2024. Except for *P. guizhouensis*, which is classified as Data Deficient, *P. kuankuoshuiensis*, *P. puxiongensis*, and *P. shuichengensis* are categorized as Critically Endangered, *P. jinbo* as Endangered, and *P. flavomaculatus* as Vulnerable (IUCN 2025). The ongoing population declines indicate the urgent need for more stringent conservation measures to ensure the long-term survival of these montane species.

Phylogenetic relationships between the genus *Pseudohynobius* and other genera of the family Hynobiidae have been resolved, and *P. puxiongensis* is recognized as the basal clade of the genus (Peng et al. 2010; Pyron and Wiens 2011; Zheng et al. 2011; Yang et al. 2013; Chen et al. 2015; Zhao et al. 2016; Dubois et al. 2021; Zhang et al. 2022). Divergence times estimated using 29 nuclear genes show that the genus originated in the middle Miocene, ca. 15.8 million years ago (Ma), with the most recent common ancestor (MRCA) occurring at 6.7 Ma (Chen et al. 2015). However, the times obtained using the mitogenome or cytochrome oxidase subunit I (COI) are much greater (Zheng et al. 2011; Zhao et al. 2016; Zhang et al. 2022). Although significant progress has been made in the phylogeny and taxonomy of this genus, the lack of comprehensive molecular phylogenetic and biogeographical analyses for all species of *Pseudohynobius* has resulted in a limited understanding of interspecific divergence times and diversification dynamics within the genus. Interestingly, four of the six species are distributed in Guizhou—namely *P. guizhouensis*, *P. jinbo*, *P. kuankuoshuiensis*, and *P. shuichengensis* (Fig. 1) (Tian et al. 2006; Zeng et al. 2006; Xu et al. 2007; Li et al. 2010; Xia et al. 2012; Li et al. 2020; AmphibiaChina 2025)—suggesting that Guizhou may represent an important region for the diversification of species within the genus.

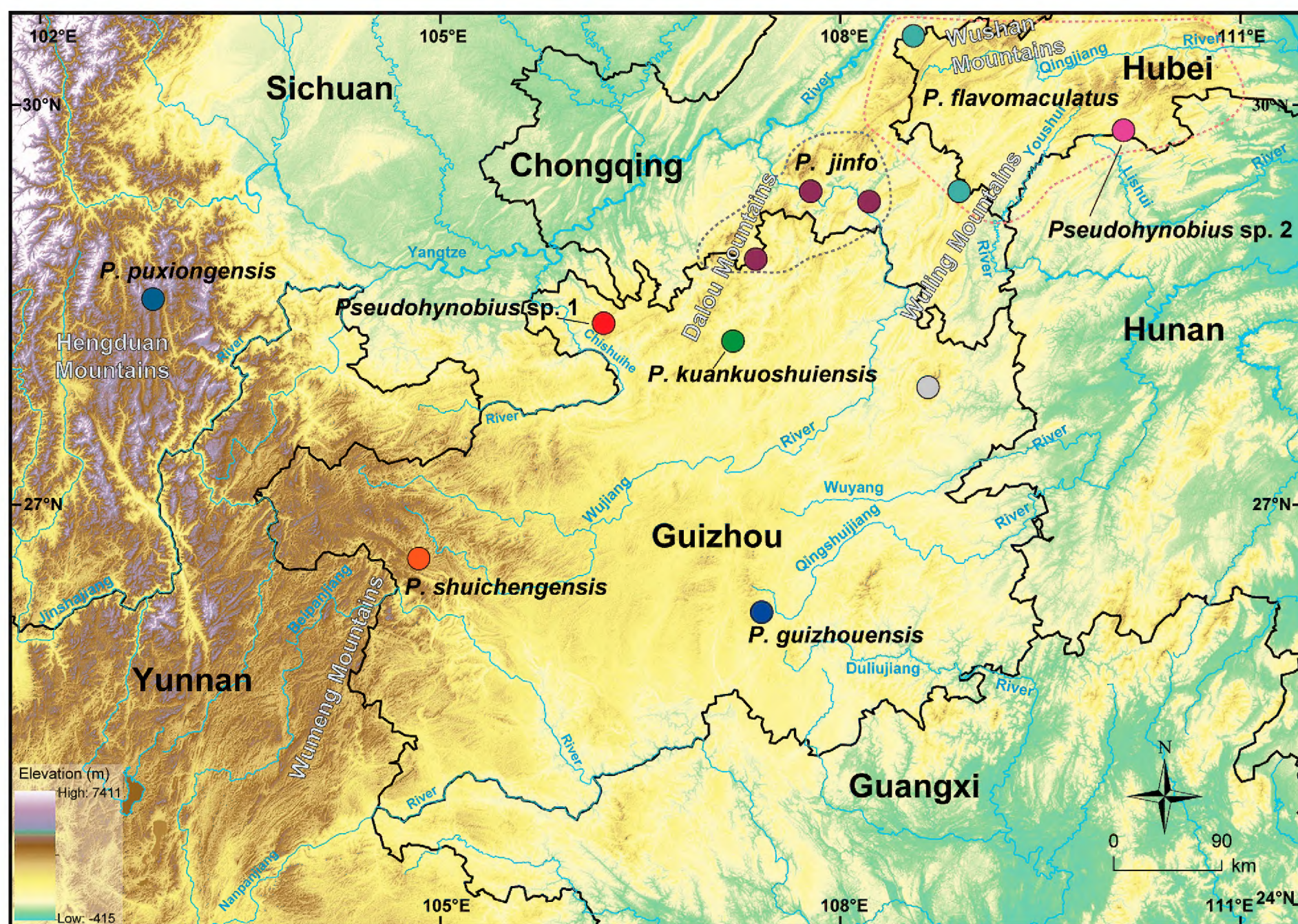


Figure 1. Geographic distribution of the six *Pseudohynobius* species. Red circles indicate newly collected samples in this study, while gray circles represent localities where the species was observed but no specimens were obtained.

In 2024, we collected a juvenile of the genus *Pseudohynobius* during a field survey in Xishui County, Guizhou Province, China (Fig. 1). A total of 16 surveys were conducted in the following near two years, none of which found an adult. To determine the phylogenetic position of this sample, we performed mitogenome sequencing. Based on the newly sequenced and published data, we (1) inferred the phylogeny and divergence timing of the genus *Pseudohynobius* and (2) examined its biogeography and evolutionary dynamics.

Materials and methods

Sampling, DNA extraction, and sequencing

A single juvenile specimen of the genus *Pseudohynobius* (Fig. 2A, B) was collected in August 2024 from Xishui County, Guizhou Province, China (Fig. 1). The habitat was a pond in a bamboo forest with a large number of subadults; no eggs or adults were found. Over the following near two years, 16 field surveys were conducted, but no additional adults or juveniles were observed. In addition, we also detected the presence of a *Pseudohynobius* population in the Fanjingshan Mountains, Guizhou Province, China, although no specimen was obtained (Fig. 2C). All experimental procedures strictly adhered to the National Standard Guidelines of the People's Republic of China (GB/T 35892-2018). Euthanasia was humanely performed using tricaine methanesulfonate (MS-222), and the specimen was subsequently preserved in the Animal Ecology Laboratory at Guizhou Normal University, Guiyang, Guizhou Province, China.

To clarify the phylogenetic placement of the juvenile *Pseudohynobius* specimen collected from Xishui County, Guizhou Province, China, we sequenced its mitochondrial genome. Total genomic DNA was extracted from each sample from 95% ethanol-preserved tissues using the cetyltrimethylammonium bromide method. Illumina sequencing libraries with 300–500 bp insert fragments were generated for each sample and sequenced on the Illumina NovaSeq 6000 sequencer at TSING KE Biological Technology Co. (Chengdu, China), generating approximately 12 Gb of raw data. The raw data were filtered using fastp v.0.23.4 (Chen 2023) using default parameters, after which the mitogenome was assembled using MitoZ v.3.6 (Meng et al. 2019).

Phylogenetic reconstruction

In this study, we used a total of 24 mitochondrial sequences from the genus *Pseudohynobius* for phylogenetic analysis, including a newly sequenced mitochondrial genome, as well as five mitochondrial genomes, 15 cytochrome *b* (Cyt *b*) sequences, two COI sequences, and two 16S rRNA sequences downloaded from GenBank (Table 1) (Fu et al. 2001; Zeng et al. 2006; Zhang et al. 2006; Peng et al. 2010; Xia et al. 2012; Yang et al. 2013; Huang et al. 2016; Zhang et al. 2022). We constructed two sequence matrices: dataset 1, which contains only Cyt *b* and is used for species delimitation and calculation of genetic distances; and dataset 2, which includes complete mitogenomes and concatenated sequences of some species (Cyt *b*, COI, and 16S rRNA) for assessing divergence times and inferring biogeography.



Figure 2. Two cryptic species of the genus *Pseudohynobius*. A, B. Specimen from Xishui County, Guizhou, China; C. Specimen photographed in Fanjingshan National Nature Reserve, Guizhou, China, by Mr. Xian-Wei Meng, who authorized its use.

Table 1. Localities, voucher information, and GenBank numbers for all samples used in this study.

ID	Species	Location (* type localities)	Voucher number	Mitogenome /Cyt b /COI/16S	References
1	<i>Pseudohynobius</i> sp. 1	Xishui County, Guizhou, China	XS02	PV018879	This study
2	<i>P. flavomaculatus</i>	Lichuan County, Hubei, China	CIB-XM2084	NC_020635	Peng et al. 2010
5	<i>P. flavomaculatus</i>	Lichuan County, Hubei, China	MVZ231151	AY028774	Fu et al. 2001
6	<i>P. flavomaculatus</i>	Lichuan County, Hubei, China	MVZ231150	AY028773	Fu et al. 2001
4	<i>Pseudohynobius</i> sp. 2	Tianping Mountain, Sangzhi County, Hunan, China	CIB-200087	DQ335719	Zeng et al. 2006
7	<i>P. guizhouensis</i>	Guiding County, Guizhou, China*	GZNU 2007110702	JX867277 JN165828 JN165944	Yang et al. 2013; Xia et al. 2012
8	<i>P. guizhouensis</i>	Guiding County, Guizhou, China*	GZNU 2007041501	JX867273	Yang et al. 2013
9	<i>P. guizhouensis</i>	Guiding County, Guizhou, China*	GZNU 2007041502	JX867274	Yang et al. 2013
3	<i>P. jinjo</i>	Pengshui County, Chongqing, China	NA	MT476485	Zhang et al. 2022
10	<i>P. jinjo</i>	Jinfo Mountain, Chongqing, China*	SWU-AMP -140715001	NC_026698	Huang et al. 2016
11	<i>P. jinjo</i>	Jinfo Mountain, Nanchuan County, Chongqing, China*	CIB-zyc825	DQ335720	Zeng et al. 2006
12	<i>P. jinjo</i>	Jinfo Mountain, Nanchuan County, Chongqing, China*	CIB-zyc826	DQ335721	Zeng et al. 2006
13	<i>P. jinjo</i>	Baima Mountain, Wulong County, Chongqing, China	CIB-bms 20010715020	DQ335724	Zeng et al. 2006
14	<i>P. kuankuoshuiensis</i>	Suiyang County, Guizhou, China	CIB-XM1073	DQ335717 EU586292 JN165945	Zeng et al. 2006; Xia et al. 2012
15	<i>P. kuankuoshuiensis</i>	Sujiagou, Suiyang County, Guizhou, China*	GZNU 2008082201	JX867278	Yang et al. 2013
16	<i>P. kuankuoshuiensis</i>	Sujiagou, Suiyang County, Guizhou, China*	GZNU 2006060801	JX867275	Yang et al. 2013
17	<i>P. shuichengensis</i>	Shuicheng County, Guizhou, China*	CIB-XM2855	NC_021001	Peng et al. 2010
18	<i>P. shuichengensis</i>	Shuicheng County, Guizhou, China*	GZNU 2007110701	JX867276	Yang et al. 2013
19	<i>P. shuichengensis</i>	Shuicheng County, Guizhou, China*	CIB-XM1078	DQ335713	Zeng et al. 2006
20	<i>P. shuichengensis</i>	Shuicheng County, Guizhou, China*	CIB-XM1079	DQ335714	Zeng et al. 2006
21	<i>P. puxiongensis</i>	Yuexi County, Sichuan, China*	CIB-XM3126	NC_020634	Peng et al. 2010
22	<i>Liua shihi</i>	NA	NA	NC_008078	Zhang et al. 2006
23	<i>Liua tsinpaensis</i>	NA	NA	NC_008081	Zhang et al. 2006
24	<i>Batrachuperus londongensis</i>	NA	NA	NC_008077	Zhang et al. 2006
25	<i>Batrachuperus pinchonii</i>	NA	NA	NC_008083	Zhang et al. 2006
26	<i>Batrachuperus tibetanus</i>	NA	NA	NC_008085	Zhang et al. 2006
27	<i>Batrachuperus yenyuanensis</i>	NA	NA	NC_012430	Zhang et al. 2006
28	<i>Salamandrella keyserlingii</i>	NA	NA	NC_008082	Zhang et al. 2006

Note: *Pseudohynobius* sp. 2 previously known as *P. flavomaculatus*. NA: Not available.

The mitogenome was extracted using PhyloSuite v.1.2.3 (Zhang et al. 2020a) for 13 protein-coding genes, two rRNAs, and 22 tRNAs for subsequent genetic analysis.

Multiple sequence alignment was performed using MAFFT v.7.4 (Kato and Standley 2013) within PhyloSuite v.1.2.3 (Zhang et al. 2020a) and checked using MEGA v.7.0 (Kumar et al. 2016) to rule out possible errors. In addition, PartitionFinder v.2.1.1 (Lanfear et al. 2017) was used to select the best-fit partitioning and nucleotide substitution model for the two datasets based on the Bayesian information criterion. In dataset 1, each gene fragment was preset as an independent partition. We followed a previous study and selected the genus *Liua* (two species), the genus *Batrachuperus* (four species), and *Salamandrella keyserlingii* as outgroups (Table 1).

For dataset 1, phylogenetic trees were reconstructed using Bayesian inference (BI) and maximum likelihood (ML) methods based on best-fit partitioning and nucleotide substitution models. Bayesian inference analysis was

performed using MrBayes v.3.2.1 (Ronquist et al. 2012). Each BI analysis was run independently using four Markov chain Monte Carlo chains (three heated chains and one cold chain) starting with a random tree; each chain was run for two million generations and sampled every 1,000 generations. Convergence of the data runs was confirmed when the average standard deviation of split frequencies was less than 0.01. The ML analysis was performed using IQ-TREE v.2.0.4 (Nguyen et al. 2015) based on the best-fit model with 10,000 ultrafast bootstrap (UFB) replicates (Hoang et al. 2018). The ML analysis was performed until a correlation coefficient of at least 0.99 was achieved.

Species delimitation and genetic distances

To infer whether the juvenile sample represents a cryptic species and to identify possible cryptic species within the genus *Pseudohynobius*, we performed species delimitation.

We employed three species delimitation methods based on the concept of phylogenetic species: Assemble Species by Automatic Partitioning (ASAP) (Puillandre et al. 2021), based on sequence variation; the Bayesian implementation of the Poisson Tree Processes (bPTP) (Zhang et al. 2013) and Multi-rate Poisson Tree Processes (mPTP) (Kapli et al. 2017), both based on phylogenetic trees. Tree-based approaches incorporate phylogenetic relationships and are particularly useful when branch lengths reflect evolutionary divergence, while ASAP focuses on pairwise genetic distances and tends to be more conservative (Zhang et al. 2013; Kapli et al. 2017; Puillandre et al. 2021). This complementary use of multiple methods allows for a more comprehensive and reliable assessment, especially in light of potential variation in sampling strategy and population history. For ASAP (<https://bioinfo.mnhn.fr/abi/public/asap>, accessed January 11, 2025), the calculations were performed online using dataset 1 and the default parameters. For bPTP (<https://species.h-its.org>, accessed January 11, 2025) and mPTP (<http://mptp.h-its.org>, accessed January 11, 2025), the calculations were performed online using the ML tree and the time tree of dataset 1, with the default parameters. Final species definition results were obtained from the consensus of the three delimitation methods.

Genetic distances were calculated using the uncorrected *p*-distance model with 1,000 bootstrap replications in MEGA v.7.0 (Kumar et al. 2016).

Divergence time estimation

Molecular dating and time tree reconstruction were conducted in BEAST v.2.4.7 (Bouckaert et al. 2014), using dataset 2. We set two calibration nodes with reference to Chen et al. (2015): (1) the divergence of *Salamandrella keyserlingii* from *Liua* and *Batrachuperus* occurred at 33.3 Ma, with a 95% confidence interval (CI) of 38.6–28.4 Ma; (2) the MRCA of the genus *Pseudohynobius* was at 6.7 Ma (95% CI: 8.7–5.0 Ma). The BEAST analysis used an uncorrelated lognormal relaxed clock and a Yule tree prior. The analysis was run for 200 million generations and sampled every 1,000 generations. All calibrations used a normal prior, monophyly, and standard deviation values of 2.7 (for calibration node 1) and 1.0 (for calibration node 2). Convergence of run parameters was checked using Tracer v.1.7.1 (Rambaut et al. 2018) to ensure that the effective sample size of all parameters was greater than 200. Three runs were performed, and the resulting trees were merged using LogCombiner v.2.4.7 (Bouckaert et al. 2014). A maximum clade credibility tree was generated using TreeAnnotator v.2.4.7 (Bouckaert et al. 2014) with a burn-in of 25%.

Ancestral area reconstruction and dynamic meta-analysis

The possible ancestral areas and dispersal routes of the genus *Pseudohynobius* were reconstructed using the

package BioGeoBEARS (Matzke 2013). Prior to analysis, six models were evaluated to select the best-fitting model (Matzke 2014), including Dispersal–Extinction–Cladogenesis (DEC), a maximum likelihood version of Dispersal–Vicariance Analysis (DIVALIKE), and a Bayesian biogeographic inference model (BAYAREALIKE), each with and without founder-event speciation (+J). The maximum number of areas in the ancestral ranges was limited to two.

To infer the divergence dynamics of the genus *Pseudohynobius*, we followed the methods outlined in a previous phylogenetic study (Li et al. 2024). Results from BEAST were summarized to obtain time intervals with 95% confidence intervals for lineage divergence events. These events were defined as the maximum number of observed lineage divergence events (MDivE) per 0.5 Ma and were used to illustrate temporal trends. The data were smoothed by calculating means using a 0.5 Ma sliding window.

Results

Sequence information and elevation distribution

The total lengths of dataset 1 and dataset 2 were 1,140 bp (640 conserved sites, 500 variable sites, and 393 parsimony-informative sites) and 15,540 bp (10,147 conserved sites, 5,351 variable sites, and 3,509 parsimony-informative sites), respectively. For dataset 1, the nucleotide substitution models for the 1st, 2nd, and 3rd codons are TVMEF+G, TRN+I, and HKY+G. The nucleotide substitution model for dataset 2 is shown in Table 2. The newly sequenced mitogenome was deposited in NCBI under accession number **PV018879**.

Table 2. Best-fit partitioning and evolutionary models of the mitogenome (dataset 2) used for phylogenetic analysis.

ID	Partition schemes	Length (bp)	Best Model
1	12S, 16S	2569	GTR+I+G
2	tRNAs	1557	HKY+G
3	Cyt b, ATP6, ND1, ND3, ND2, ND4, ND5, ATP8, ND4L	7863	TVM+I+G
4	COI, COII, COIII	3032	GTR+I+G
5	ND6	519	HKY+I

Species of the genus *Pseudohynobius* show a west-to-east distribution pattern in southwestern China, along the Hengduan Mountains, the Wumeng Mountains, the Dalou Mountains, and the Wuling–Wushan Mountains (Fig. 1). The overall trend in elevation decreases from west to east, with a mean elevation of about 1,750 m. Among them, *P. puxiongensis*, *P. shuichengensis*, and *P. jinbo* are distributed above 1,750 m, while *P. flavomaculatus*, *P. guizhouensis*, *P. kuankuoshuiensis*, and the two cryptic species occur below this elevation (Fig. 3).

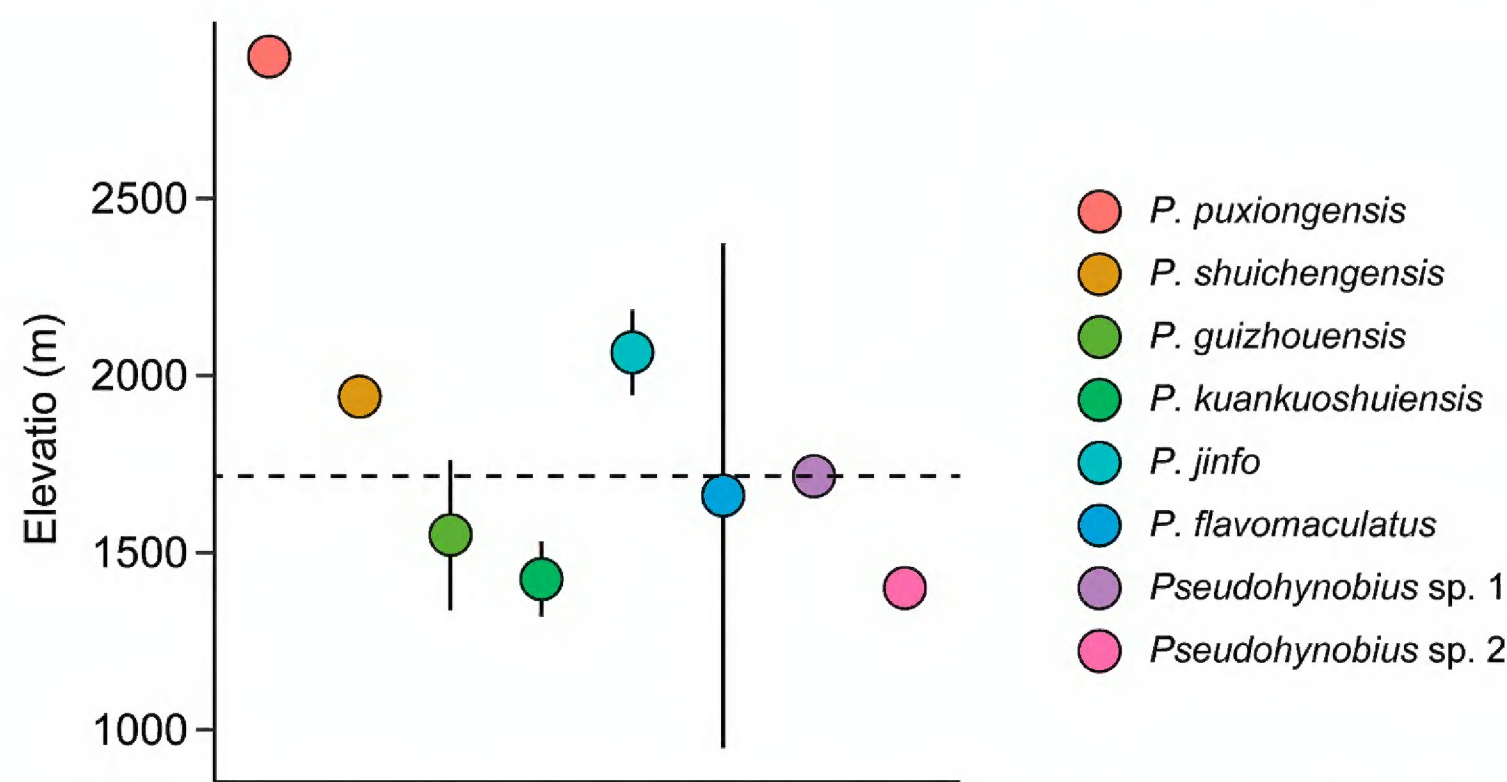


Figure 3. Elevational ranges of species of the genus *Pseudohynobius*, with dashed lines indicating mean elevations.

Species delimitation, phylogeny, and genetic distance

Species delimitation based on 28 *Cyt b* sequences using three methods (ASAP, bPTP, and mPTP) assigned the sequences to eight phylogenetic species within *Pseudohynobius* (Suppl. materials 1–3). The newly collected single sample and *P. flavomaculatus* (DQ335719) were identified as independent phylogenetic species, designated *Pseudohynobius* sp. 1 and *Pseudohynobius* sp. 2, while *P. flavomaculatus* (MT476485) clustered with four samples of *P. jinfo* (Suppl. materials 1–3).

Both maximum likelihood and Bayesian analyses of dataset 1 produced the same topology (Fig. 4, Suppl. material 4). The BI and ML trees showed similar branch lengths and support values; within *Pseudohynobius*, the BI tree had 16 internal nodes with posterior probability support > 0.95 (Fig. 4A), and the ML tree had 12 internal nodes with bootstrap support > 95% (Fig. 4B). For species-level clades, low support was observed only between *P. kuankuoshuiensis* and two other species (*Pseudohynobius* sp. 1 and *P. guizhouensis*). *Pseudohynobius* can be divided into three clades: Clade I, the basal clade, includes only *P. puxiongensis*; Clade II contains *P. flavomaculatus*, *Pseudohynobius* sp. 2, and *P. jinfo*; and Clade III includes *P. shuichengensis*, *P. kuankuoshuiensis*, *Pseudohynobius* sp. 1, and *P. guizhouensis* (Fig. 4). A similar topology was obtained for the BI tree reconstructed from dataset 2 (Fig. 5), but it showed distinct inconsistencies between *P. shuichengensis* and *P. kuankuoshuiensis* (Suppl. material 5).

Genetic distances based on *Cyt b* revealed that the two cryptic lineages exhibit minimum interspecific distances of 3.2% and 4.2% compared to other congeners (Table 3), which are substantially higher than the recently reported minimum distance of 2.1% between *Hynobius chinensis* and *H. guabangshanensis* (Wang et al. 2023). This indicates that genetic differentiation between the two cryptic lineages and their closely related species has reached the species level.

Table 3. Uncorrected *p*-distance between *Pseudohynobius* species for the mitochondrial *Cyt b* gene.

ID	Species	1	2	3	4	5	6	7
1	<i>Pseudohynobius</i> sp. 1							
2	<i>Pseudohynobius</i> sp. 2	12.7						
3	<i>P. flavomaculatus</i>	13.0	4.2					
4	<i>P. guizhouensis</i>	3.2	12.7	13.1				
5	<i>P. jinfo</i>	14.3	6.1	7.0	14.7			
6	<i>P. kuankuoshuiensis</i>	9.8	12.4	12.4	10.1	13.2		
7	<i>P. puxiongensis</i>	13.5	15.1	14.8	14.1	16.2	14.4	
8	<i>P. shuichengensis</i>	10.4	12.3	11.8	10.3	13.0	10.5	14.6

Divergence time and biogeography

Our molecular dating results suggest that *Pseudohynobius* originated in the middle Miocene, approximately 14.57 Ma (95% CI: 19.54–10.32) (node 1), and that the extant crown group originated in the late Miocene, approximately 8.62 Ma (95% CI: 10.48–6.80) (node 2) (Fig. 5A). Interspecific divergence occurred primarily between 8 Ma and 1.4 Ma (Fig. 5A).

Changes in clade divergence within *Pseudohynobius* were assessed over time using biogeographic dynamic meta-analysis. In total, nine clade divergence events were identified (Suppl. material 6). MDivE analyses showed that cladistic divergence began at approximately 10 Ma, increased rapidly between 8 and 5 Ma, peaked around 4 Ma, and declined sharply after 2 Ma (Fig. 5B).

The results of ancestral area reconstruction are shown in Fig. 5, Suppl. material 4. Models with “+J” had higher log-likelihood (LnL) values than those without, indicating a significantly better fit in all cases except for DIVALIKE (Table 4). Under the best-fit DEC+J model, vicariance and dispersal events likely shaped the current diversity and distribution of *Pseudohynobius*. Ancestral area reconstruction under this model suggests that the most recent common ancestor of extant *Pseudohynobius* most likely inhabited the Hengduan Mountains and the Dalou Mountains (Fig. 5A). An initial vicariant event separated the ancestral populations into Clade I and Clades II–III,

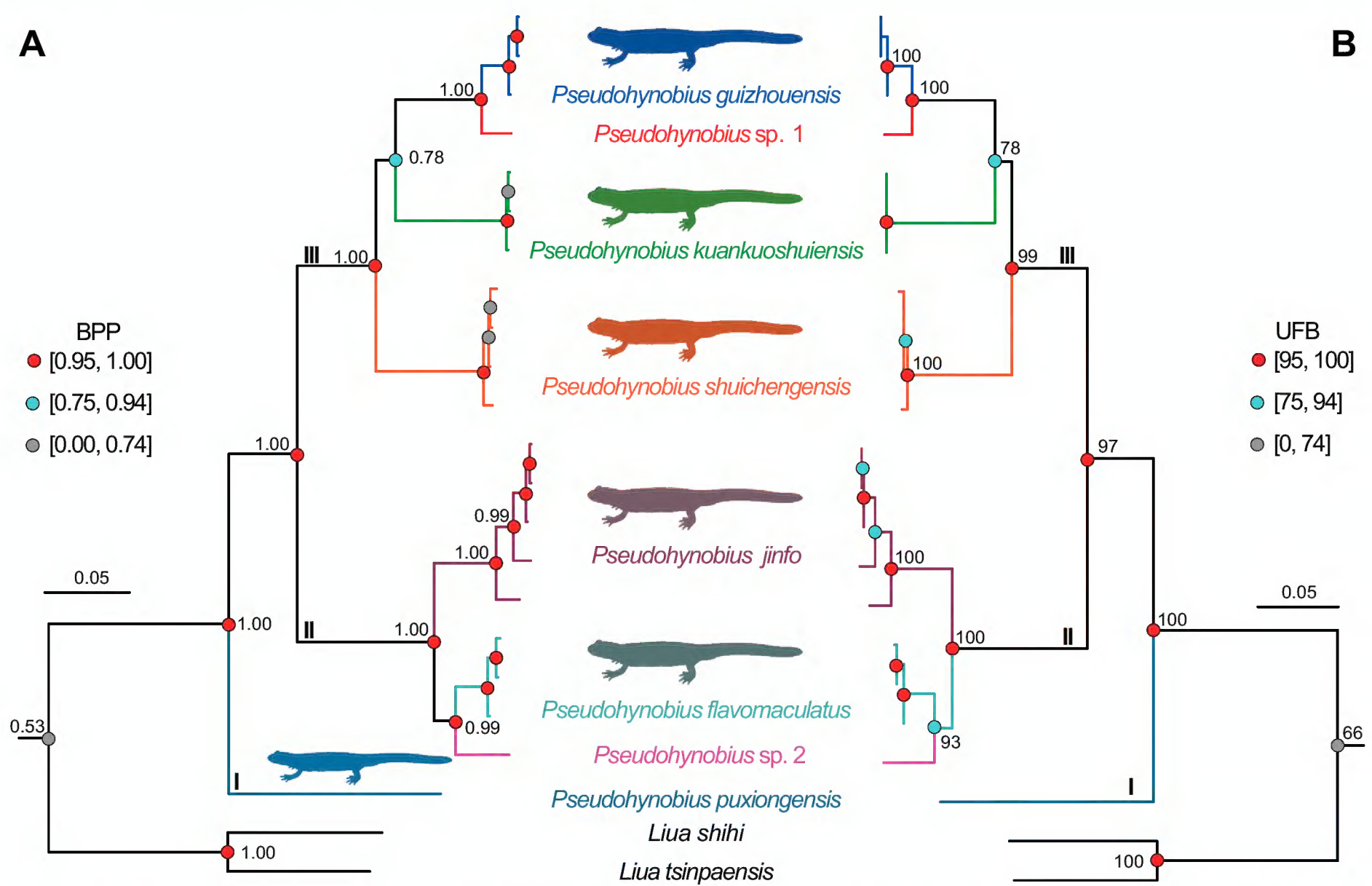


Figure 4. Phylogeny and species delimitation based on mitochondrial *Cyt b*. **A.** BI tree; Bayesian posterior probabilities (BPP) from BI analyses are shown at nodes; **B.** ML tree; ultrafast bootstrap supports (UFB) from ML analyses are shown at nodes. Branches are color-coded to represent different species based on the consensus results of species delimitation using ASAP, bPTP, and mPTP.

Table 4. Estimated parameters and statistical results for six models (DEC, DIVALIKE, BAYAREALIKE, and their corresponding +J models) using the R package BioGeoBEARS. The model with the highest AIC model weight is considered the best model and is shown in bold. Abbreviations: LnL, log-likelihood; *d*, rate of dispersal; *e*, rate of extinction; *j*, likelihood of founder-event speciation at cladogenesis; AIC, Akaike’s information criterion.

Model	LnL	Number of parameters	<i>d</i>	<i>e</i>	<i>j</i>	AIC	AIC model weight
DEC	-17.72	2	0.027	0.034	0	39.44	0.003
DEC+J	-11.51	3	1.00E-12	1.00E-12	0.15	29.03	0.550
DIVALIKE	-14.24	2	0.021	1.00E-12	0	32.49	0.098
DIVALIKE+J	-14.24	2	0.021	1.00E-12	0	32.49	0.098
BAYAREALIKE	-21.57	2	0.048	0.160	0	47.14	6.40E-05
BAYAREALIKE+J	-12.30	3	1.00E-07	1.00E-07	0.16	30.60	0.250

after which the ancestral populations in the Dalou Mountains dispersed into the Wumeng Mountains, the Wuling–Wushan Mountains, and the Miaoling Mountains at 4.71 Ma, 3.19 Ma, and 1.44 Ma, respectively (Fig. 5A). In situ diversification in the Dalou and Wuling–Wushan Mountains may also have contributed to species formation, as in the cases of *P. jinbo* and *P. flavomaculatus* (Fig. 5A).

Discussion

Phylogeny and underestimated diversity of the genus *Pseudohynobius*

Our phylogeny is consistent with the results of Yang et al. (2013): *P. puxiongensis* represents the basalmost clade, Clade I; *P. flavomaculatus* and *P. jinbo* comprise Clade II;

and *P. guizhouensis*, *P. kuankuoshuiensis*, and *P. shuichengensis* comprise Clade III. However, we also discovered that *P. kuankuoshuiensis* is only moderately supported as the sister clade of *P. guizhouensis*–*Pseudohynobius* sp. 1 (Figs 4, 5). DensiTree plots, in which the topologies of multiple time trees were superimposed, showed discordance between *P. kuankuoshuiensis* and *P. shuichengensis* (Suppl. material 5). We hypothesize that gene flow may be the main contributor to this discordance. Similar discordance was observed between *Pseudohynobius*, *Liua*, and *Batrachuperus*, which have a phylogenetic relationship of (*Liua*, (*Batrachuperus*, *Pseudohynobius*)) (Figs 4, 5) or (*Pseudohynobius*, (*Liua*, *Batrachuperus*)) (Zhang et al. 2022), but the nuclear gene tree supports a topology of (*Batrachuperus*, (*Pseudohynobius*, *Liua*)) (Chen et al. 2015). In addition, we also took into account the taxonomic controversy mentioned in the introduction, as it may influence

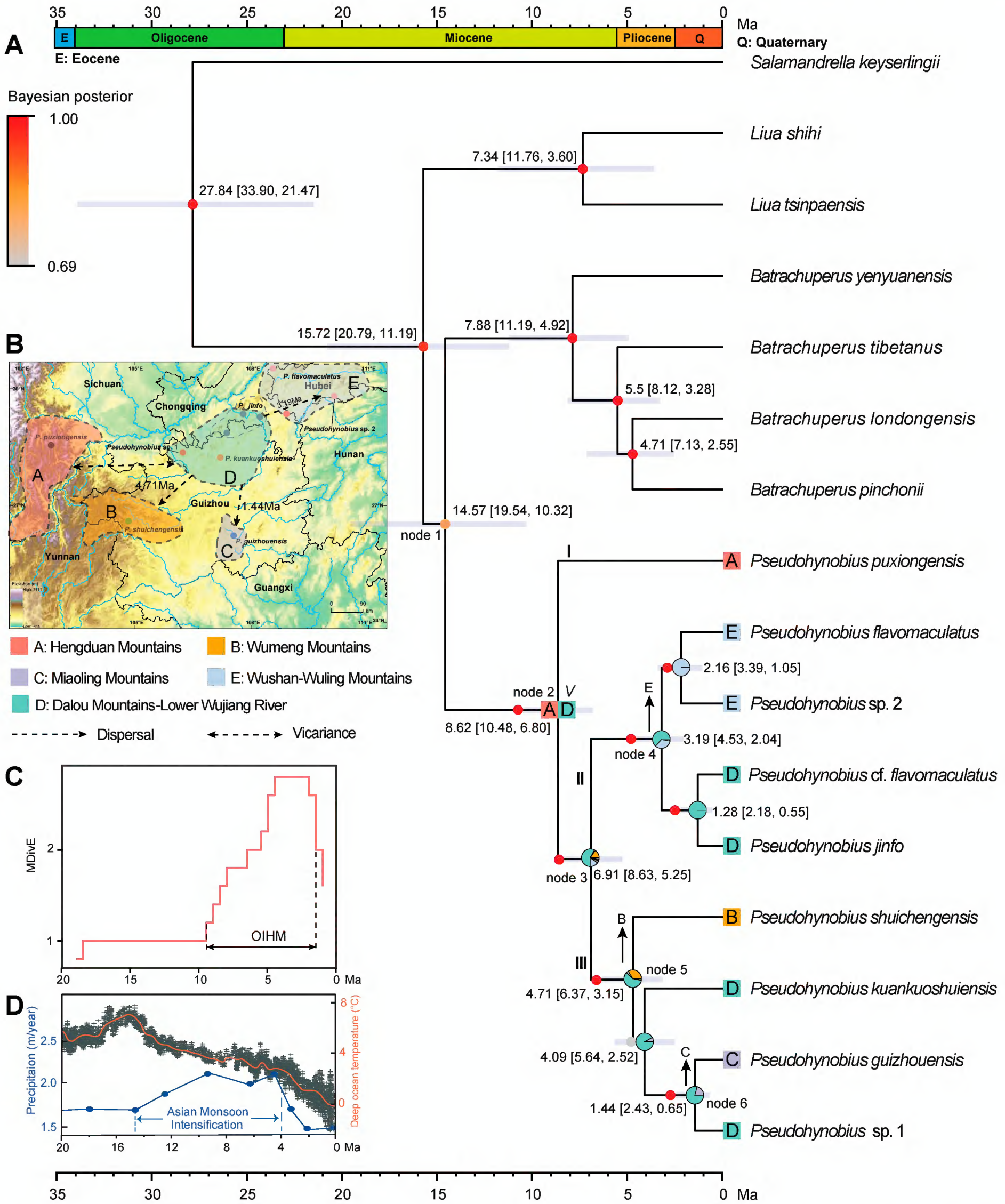


Figure 5. Time tree, biogeographic history, and diversification dynamics based on mitogenomes. **A.** Time tree with ancestral area reconstruction. Blue bars at nodes indicate 95% CI of divergence times. Circled nodes represent the most probable ancestral regions; arrows show dispersal directions; **B.** Possible dispersal routes of the genus *Pseudohynobius*; **C.** Divergence dynamics based on the maximum number of observed lineage divergence events (MDivE) per Ma. OIH: orogeny intensification of the Hengduan Mountains; **D.** Global temperature inferred from deep-sea oxygen isotope records ($\delta^{18}\text{O}$) in benthic foraminifera (Zachos et al. 2008), modeled mean annual precipitation under idealized CO_2 levels (Farnsworth et al. 2019).

the interpretation of phylogenetic and biogeographic reconstruction results. Evidence has been presented that gene flow, incomplete lineage sorting, and hybridization may all contribute to this discordance (Meleshko et al. 2021; Rivas-González et al. 2023), but the major contribution remains to be supported by phylogenomic evidence.

Integrating taxonomy for species delimitation is becoming a consensus, where phylogenetic species should be accepted when evidence of phylogenetic isolation is supported by other results, e.g., morphology, reproduction, and climatic niche (van Elst et al. 2025). In this study, phylogeny (Fig. 3), species delimitation (Suppl. materials 1, 2), and genetic distance (Table 3) support the existence of two cryptic phylogenetic species in *Pseudohynobius*: *Pseudohynobius* sp. 1 from Xishui, Guizhou (Fig. 2A, B), and *Pseudohynobius* sp. 2 from Tianping Mountain, Sangzhi County, Hunan. We also acknowledge that for the two identified cryptic lineages, species delimitation was based on Cyt *b* sequences from only a single sample, and no morphological comparisons were possible due to the lack of adult specimens and detailed morphological data. This limitation may introduce additional bias. Therefore, future efforts that expand field surveys to include more specimens, incorporate nuclear gene fragments or even genome-wide data, and obtain sufficient morphological evidence—particularly from adult individuals—will be essential to provide more robust support for species delimitation.

In addition, further surveys are needed in the Dalou Mountains, Wuling Mountains, and Wushan Mountains. For example, our 2022 photographs of unknown species of the genus *Pseudohynobius* in the Fanjingshan Mountains (Figs 1, 2C), combined with the two new cryptic species revealed in this study, suggest that the range and diversity of the genus *Pseudohynobius* need to be reassessed, which is valuable for formulating conservation policies and carrying out conservation actions.

High-elevation speciation dominated by orogeny

For the mitogenome, the estimated origination time for the total group of *Pseudohynobius* (14.57 Ma vs. 49.85 Ma) and for crown group *Pseudohynobius* (8.62 Ma vs. 33.38 Ma) are younger than those reported by Zhang et al. (2022). The reasons for generating older divergence times are related to alternative saturation and the rapid evolution of mitochondrial DNA, resulting in rather ancient estimates (Zheng et al. 2011; Chen et al. 2015). However, we cannot simply assume that divergence times assessed using mitogenomes are always less precise than those derived from nuclear markers or that mitochondrial dates are always overestimated (Chen et al. 2015). When divergence times are less than 15 Ma, mitochondrial substitutional saturation is minimal, and estimates based on mitochondrial data can still be precise (Chen et al. 2015). Our origin and interspecific divergence times are in close agreement with the results of Chen et al. (2015) and are less than 15 Ma, so it can be assumed that it is reasonable for us to infer the timing of the origin and divergence of the genus *Pseudohynobius*.

Orogeny and low temperatures have promoted divergence and diversification within the genus *Pseudohynobius*. Orogeny creates a variety of environmental conditions, including climatic niches, new habitats, and dispersal barriers, while low temperatures may isolate

populations in specific habitats—all of which are conducive to promoting speciation (Hoorn et al. 2013; Xing and Ree 2017; Rahbek et al. 2019a; Li et al. 2021). Our inferred MDivE curve (Fig. 5C) shows that the divergence rate of *Pseudohynobius* was high between 9 and 4 Ma, and the diversification trend coincides with the rapid uplift of the Hengduan Mountains and the Yunnan–Guizhou Plateau from the late Miocene to late Pliocene (Zhou and Chen 1993; Sun et al. 2011; Favre et al. 2015), suggesting that the diversification of *Pseudohynobius* may be closely linked to orogenic events in these regions.

As part of the continued expansion of orogeny—such as the uplift of the Qinghai–Tibet Plateau (Ding et al. 2022) and the right-lateral extrusion of Indochina (Zhang et al. 2009; Fyhn and Phach 2015)—the Hengduan Mountains and the Yunnan–Guizhou Plateau experienced rapid uplift during the late Miocene, peaking just before the late Pliocene (Zhou and Chen 1993; Zhang et al. 2009; Wang et al. 2012). Strong orogeny shaped high-elevation mountains and varied microhabitats, which may have driven differentiation of ecological niches among populations (Rahbek et al. 2019b). Topography and microclimatic changes may have led *Pseudohynobius* to favor narrow, high-altitude cold regions, thereby promoting adaptation and speciation at high elevations (Fig. 2) (Fei and Ye 2016). For instance, in *P. jinbo*, the capillaries are in close contact with and protrude toward the epidermis, while the dorsal pigmentation layer is more developed (Jing et al. 2017). Such features in the high-altitude frog *Nanorana parkeri* have been linked to adaptations to hypoxia and elevated ultraviolet radiation at high altitudes (Yang et al. 2019; Fu et al. 2022).

Topographic changes influence the formation and migration of rivers, which may also act as barriers to gene flow between distinct populations. At present, the Jinshajiang River, Wujiang River, and Qingshuijiang River serve as geographical boundaries separating the distributions of species within the genus (Fig. 1). U-Pb dating evidence based on detrital zircon grains suggests that the establishment of the modern Yangtze River occurred in the late Miocene, from about 11 to 5 Ma (Fu et al. 2021; He et al. 2023), which is consistent with the divergence of the basal clade *P. puxiongensis* from 10.48 to 6.8 Ma (Fig. 4). Interspecific divergence within Clade II, on the other hand, corresponds to the formation of the Wujiang and Miaoling Mountains during the Pliocene to early Pleistocene (Zhou and Chen 1993), which may have served as geographic barriers contributing to species divergence. Distribution patterns of batrachians inhabiting southwestern China are severely affected by orogeny and riverine barriers, with close examples including *Batrachuperus yenyuanensis* (Jia et al. 2019), *Glyphoglossus yunnanensis* (Zhang et al. 2020b), and the *Paramesotriton caudopunctatus* complex (Chen et al. 2011; Luo et al. 2021). In addition, the intensification of the Asian monsoon may have promoted plant growth and geomorphological erosion in southwestern China (Fig. 4C), thereby providing abundant food resources and geographic isolation (Antonelli et al. 2018; Ding et al. 2020), which in turn created new ecological and evolutionary opportunities for speciation.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by the 2024 National Key Protected Wildlife Resources Survey in Xishui County, Guizhou Province, China (11904/0624063), the 2024 National Key Protected Wildlife Resources Survey in the Chishui River Basin, Guizhou (P52000020240003VQ), and the Research Project on the Reproductive Biology of the Hainan Gibbon (KY-2000105-KT).

Authors' contributions

Tao Luo, Jiang Zhou, and Huai-Qing Deng conceived and designed the research; Tao Luo, Zhong-Lian Wang, and Zi-Fa Zhao conducted field surveys and collected samples; Tao Luo and Ming-Yuan Xiao analyzed genetic data; and Tao Luo, Ning Xiao, and Jiang Zhou wrote and revised the manuscript. All authors have read and approved the final version of the manuscript.

Acknowledgments

We are grateful to Chang-Ting Lan and local residents who helped in the fieldwork. We are grateful to LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.

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Supplementary material 1

Species delimitation results using ASAP

Authors: Tao Luo, Zi-Fa Zhao, Zhong-Lian Wang, Ming-Yuan Xiao, Ning Xiao, Huai-Qing Deng, Jiang Zhou

Data type: png

Explanation note: Text.

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Supplementary material 2

Species delimitation results using bPTP

Authors: Tao Luo, Zi-Fa Zhao, Zhong-Lian Wang, Ming-Yuan Xiao, Ning Xiao, Huai-Qing Deng, Jiang Zhou

Data type: png

Explanation note: Text.

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Supplementary material 3

Species delimitation results using mPTP

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Data type: png

Explanation note: Text.

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Supplementary material 4

Phylogenetic analysis and species delimitation of the raw tree topology

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Data type: png

Explanation note: Text.

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Supplementary material 5

DensiTree plot of mitochondrial genome-based time tree reconstruction

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Data type: png

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Link: <https://doi.org/10.3897/zse.101.153576.suppl5>

Supplementary material 6

The inferred lineage divergence events associated with the *Pseudohynobius* and their ages

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Data type: docx

Explanation note: Text.

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